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Increase in cytokine production (IL-1β, IL-6, TNF-α but not IFN-γ, GM-CSF or LIF) by stimulated whole blood cells in postmenopausal osteoporosis

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Abstract

Postmenopausal osteoporosis is a progressive disorder characterized by a decreased bone mass and increased susceptibility to fractures. Several investigations have suggested that one of the mechanisms through which estrogen prevents bone loss was a modulation on secretion or release of various cytokines that are known to influence bone remodeling, even if some recent data have challenged this hypothesis. However, in established osteoporosis, the possibility that enhanced cytokines activity may account for the progression of this disease remains unclear and controversial. We sought here to determine whether production of IL-1β, IL-6, TNF-α, IFN-γ, GM-CSF and LIF, after direct stimulation in whole blood, was different in healthy ($n = 30$) or osteoporotic postmenopausal women ($n = 24$) and whether lumbar bone density (l-BMD) correlated with the values of cytokine production observed in these conditions. A significant difference was observed between the osteoporotic and control subjects for IL-1β ($p < 0.0001$), IL-6 ($p < 0.001$) and TNF-α ($p = 0.027$) productions, the values being higher in the osteoporotic women. No significant differences between the groups were observed for IFN-γ ($p = 0.51$), GM-CSF ($p = 0.70$) or LIF ($p = 0.97$). In the whole population, statistically significant negative correlations were observed between lumbar BMD and IL-1β ($r = -0.46$) ($p < 0.0005$), IL-6 ($r = -0.50$) ($p < 0.0001$) and TNF-α ($r = -0.39$) ($p < 0.005$) production while no such correlations were observed for IFN-γ, GM-CSF or LIF. In conclusion, the study of cytokine production by immune cells cultured in autologous whole blood suggests that in women more than 10 years past the menopause and presenting a decrease in lumbar bone density corresponding to the new WHO definition of

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'osteoporosis', production of IL-1β, IL-6 and TNF-α is still increased compared to controls matched for age and ovarian function, while no differences are reported for IFN-γ, GM-CSF or LIF production.

Keywords: Cytokines; Osteoporosis; Menopause; Interleukin; Densitometry

1. Introduction

Postmenopausal osteoporosis is a progressive disorder characterized by a decreased bone mass and increased susceptibility to fractures. Estrogen deficiency occurring after natural or surgically induced menopause leads to an uncoupling between activity of bone resorbing cells (osteoclasts) and bone forming cells (osteoblasts) responsible for an accelerated loss of bone [1]. Recent studies have suggested that the increase in bone resorption induced by estrogen deficiency in postmenopausal osteoporotic women is, at least partly, mediated by increased paracrine production of bone resorbing cytokines [2]. Interleukin-1 (IL-1) is one of the most potent stimulators of bone resorption [3] and Interleukin-6 (IL-6) appears to be a potent osteotropic factor that may play an important role in diseases characterized with increased bone resorption [4,5]. Tumor necrosis factor alpha (TNF-α), as IL-1, enhances bone resorption by stimulating development of osteoclast progenitors and increasing the activity of mature cells [6]. Granulocyte-macrophage colony stimulating factor (GM-CSF) may also function as a potent regulator of bone resorption since, besides its ability to stimulate IL-1 and TNF-α secretion in monocytes and neutrophils [7], it appears to stimulate osteoclast recruitment and differentiation [8,9]. Leukemia inhibitory factor (LIF) has been reported to affect bone metabolism, but results express some variability depending on the use of different models. While this cytokine is generally considered as an enhancer of bone resorption [10], recent reports suggest that LIF may exhibit an inhibitory action on the development of osteoclasts, probably through an effect on the osteogenic cells [11]. On the other hand, Interferon gamma (IFN-γ) inhibits the process of IL-1 stimulated bone resorption [3]. Therefore, it is generally accepted that one of the mechanisms through which estrogen prevents bone loss was a modulation on secretion or release of these various cytokines that are known to influence bone remodeling [8,12–14], even if some recent data have challenged this hypothesis [15]. However, in established osteoporosis, the possibility that enhanced cytokines activity may account for the progression of this disease remains unclear and controversial. Interleukin-1 alpha (IL-1α), Interleukin-1β (IL-1β) and IL-6 mRNAs were expressed significantly more often in bone samples from postmenopausal women with osteoporotic fractures than in postmenopausal women with normal bone density [16]. However, IL-1β and IFN-γ production by peripheral blood mononuclear cells and the distribution of the various subsets of these cells in the peripheral blood were reported to be independent of bone mass in osteoporotic women [17]. More recently, circulating levels of IL-1α, IL-1β and IL-6 have been found to be not significantly higher in osteoporotic women than in the normal women [18] and these values did not correlate with markers of bone turnover in healthy postmenopausal women [15].

We have previously reported that stimulation of immune cells, cultured in autologous whole blood, may be the most appropriate milieu for studying cytokine production in vitro [19]. We sought here to determine whether production of IL-1β, IL-6, IFN-γ, GM-CSF and LIF, after direct stimulation in whole blood, was different in healthy or osteoporotic postmenopausal women and whether lumbar bone density correlated with the values of cytokine production observed in these conditions.

2. Material and methods

Our study comprised 54 caucasian postmenopausal women consecutively consulting in an outpatient setting specialized in screening and prevention of metabolic bone diseases and fulfilling the

Table 1
Characteristics of cytokine immunoassays (ELISA)

Cytokine studied	Sensitivity	Precision (%)	Reproducibility (%)	Accuracy (%)
IL-1β	2 pg/ml	2.8	4.5	102
IL-6	3 pg/ml	5.1	4.8	89.2
TNF-α	3 pg/ml	3.4	9.2	97
IFN-γ	0.03 IU/ml	3.5	7.9	100
GM-CSF	3 pg/ml	4.4	9.6	103
LIF	0.4 IU/ml	4.5	6.6	102

following criteria (Bone Metabolism Unit, University Hospital of Liege). All of them were free of any disease able to interfere with bone metabolism such as renal, hepatic, inflammatory, malignant or immune disorders. Since their menopause, they never received significant amounts of drugs commonly prescribed for prevention or treatment of osteoporosis, like estrogens, calcitonin, bisphosphonates fluoride salts, anabolic steroids, vitamin D or drugs known to interact with cytokine production such as corticosteroids, non-steroidal anti-inflammatory drugs or immunomodulators.

Bone mineral density (BMD) of the lumbar spine (L2–L4) was measured by isotopic dual photon absorptiometry following the method described by Krolner and Porsnielsen [20] and which, in our hands, is characterized by an intra- and inter-assay coefficient of variation of 1.1% and 1.3%, respectively [21]. Women with an anterioposterior spine density below 2.5 S.D. from the mean of young adults (0.70 g/cm^2) were classified in the osteoporotic group ($n = 24$) [22]. This value of 0.70 g/cm^2 also corresponds to the fracture threshold previously defined for our population as the 90th percentile of the distribution of the lumbar BMD values in women suffering of vertebral crush fractures [23,24]. Women with a lumbar BMD above this value were considered as the control group ($n = 30$). In all patients ($n = 54$), the diagnosis of 'severe osteoporosis' [22] was excluded by (a) no history of fracture of the distal forearm or hip and (b) absence of vertebral fractures on lateral radiographs of the thoracic and lumbar spine.

Blood samples were collected with endotoxin free vacuum blood collection tubes containing heparin (120 IU/tube) (Kabitube®, KABI, Upsala, Sweden) and processed after a maximum storage period of 5 h at room temperature. As previously described [19], blood was diluted 1/10 in RPMI (Gibco, Gent, Belgium) complemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and was distributed in 2-ml wells. Polyclonal activators were then added: phytohemagglutinin (PHA HA16 from Wellcome Diagnostic, Dartford, UK) at a final concentration of 5 μg/ml and lipopolysaccharide (LPS from Salmonella enteritis, SIGMA, St Louis, MO) at a final concentration of 25 μg/ml. The plates were then incubated at 37°C with 5% CO_2 atmosphere.

The contents of the wells were removed at 24 or 72 h, without adding new culture medium, then centrifuged at 800 × g for 10 min to discard the cells. The supernatants were collected and frozen at −20°C until assay for cytokines.

The cytokines IL-1β, IL-6, TNF-α (in 24-h culture), IFN-γ, GM-CSF and LIF (in 72-h culture) were measured by using specific immunoassays from Medgenix Diagnostics (Medgenix Diagnostics S.A., Fleurus, Belgium). The method used, type of tracer, sensitivity, precision, reproducibility and accuracy were as described previously [15] and are summarized in Table 1.

Results were expressed as mean ± S.D. Cytokine distributions being highly skewed, a logarithmic transform was applied to normalize the distribution. All subsequent calculations were done on the transformed data. Groups were compared by Student t-test and correlations were computed to measure the association between the variables studied. Results were considered to be significant at $p < 0.05$.

Table 2
Mean production ± S.D. and range of IL-1β, IL-6 and TNF-α in control and osteoporotic postmenopausal women

Cytokine studied	Duration of culture (h)	Controls mean ± S.D. (range) ($n = 30$)	Osteoporotics mean ± S.D. (range) ($n = 24$)	p-value
IL-1β (pg/ml)	24	1390 ± 540 (543–2504)	2320 ± 880 (663–4228)	<0.0001
IL-6 (pg/ml)	24	14 700 ± 4810 (8180–25 350)	21 920 ± 8310 (10 510–42 590)	<0.001
TNF-α (pg/ml)	24	12 340 ± 5250 (6070–25 210)	15 900 ± 6560 (8510–30 320)	0.027

3. Results

Osteoporotics and controls did not differ with respect to age (64.3 ± 7.3 vs. 62.2 ± 5.9 years) and to time since menopause (14.2 ± 7.1 vs. 13 ± 6.5 years). Likewise, estradiol levels were similar in both groups: 10 ± 3.2 pg/ml in osteoporotics and 13.3 ± 9.2 pg/ml in controls. The average of lumbar BMD was 0.62 ± 0.5 g/cm² (T-score: −0.8 ± 0.5) in the osteoporotic group and 0.78 ± 0.7 g/cm² (T-score: 0.8 ± 0.7) in the control ($p <$ 0.0001).

The values of production of the different cytokines are summarized in Tables 2 and 3. A significant difference was observed between the osteoporotic and control subjects for IL-1β ($p <$ 0.0001), IL-6 ($p < 0.001$) and TNF-α ($p = 0.027$) productions, the values being higher in the osteoporotic women (Table 2). No significant differences between the groups were observed for IFN-γ ($p = 0.51$), GM-CSF ($p = 0.70$) or LIF ($p = 0.97$) (Table 3). In the whole population, statistically significant negative correlations were observed between lumbar BMD and IL-1β ($r =$ −0.46) ($p < 0.0005$) (Fig. 1), IL-6 ($r = -0.50$) ($p < 0.0001$) (Fig. 2) and TNF-α ($r = -0.39$) ($p <$ 0.005) (Fig. 3) production while no such correlations were observed for IFN-γ, GM-CSF or LIF.

Women 10 years or more past the menopause accounted for 70.8% of the osteoporotic group and 66.7% of the control group ($p > 0.1$).

4. Discussion

We tested the hypothesis that, in a population of postmenopausal osteoporotic women, selected on the basis of the latest World Health Organization's definition [22], the production of different cytokines known to modulate bone turnover was altered compared to a population of healthy controls. We used the experimental model of analysis of cytokine production by cells present in whole blood following exposure to polyclonal activators. This method was previously applied to the evaluation of the cytokine production in rheumatoid arthritis and osteoarthritis [25]. The present study is the first attempt to apply this methodology to postmenopausal osteoporosis.

Under these conditions, we observe a higher production of IL-1β, IL-6 and TNF-α in osteoporotic women while no differences appear for IFN-γ, GM-CSF or LIF. Furthermore, a negative relationship has been established between the lumbar BMD of our postmenopausal women and the production of IL-1β, IL-6 or TNF-α.

The role of IL-1 and IL-6 as mediators of the deleterious effect of estrogens withdrawal on the skeleton has been frequently debated over the last years. Pacifici et al. reported that circulating mononuclear cells from subjects with 'high turnover' osteoporosis and from early postmenopausal women, in whom bone remodeling is higher than normal, produce increased amounts of IL-1 [26] and that estrogen replacement therapy blocks this increased production of IL-1 [12]. The same authors showed that estrogens modulate the secretion of IL-1 receptor antagonist (IL-1ra) which increases with time in healthy postmenopausal women but not in osteoporotics [27]. However, these authors also suggested that products of bone resorption stimulate IL-1 release by PBMC and, therefore, that the enhanced IL-1 activity observed in states of high bone turnover could be regarded as a consequence, rather than a cause, of increased bone resorption [28]. IL-6, in

Table 3
Mean production ± S.D. and range of IFN-γ, GM-CSF and LIF in control and osteoporotic postmenopausal women

Cytokine studied	Duration of culture (h)	Controls mean ± S.D. (range) ($n = 30$)	Osteoporotics mean ± S.D. (range) ($n = 24$)	p-value
IFN-γ (IU/ml)	72	2210 ± 1510 (520–6950)	2270 ± 1060 (290–3420)	0.51
GM-CSF (pg/ml)	72	3630 ± 1840 (1660–7490)	3910 ± 2310 (940–6710)	0.70
LIF (IU/ml)	72	235 ± 111 (66.8–435.7)	218 ± 123 (3.7–466.1)	0.97

combination with IL-3 stimulates the development of the colony forming unit-granulocyte/monocyte (CFU-GM) [29], the committed precursor of bone-resorbing osteoclasts [30] and stimulates early osteoclast precursor formation from cells present in CFU-GM colonies [31]. Furthermore, high concentrations of IL-6 appear capable of stimulating mature osteoclasts to resorb bone, although this remains controversial [4]. Based on the observation that estrogens inhibit IL-6 production in cells of the osteoblastic lineage [32–36] and that an IL-6 antibody prevents the ovariectomy-induced increase in osteoclastogenesis in the bone marrow of the mouse [5,37], Manolagas et al. suggested that an inhibitory control of estrogens on the production and/or action of IL-6 in response to local and systemic bone-resorbing agents may be a possible mechanism explaining the protective effect of sex steroids against osteoporosis [37]. Inhibition of IL-6 release from human osteoblasts by estradiol was not, however, consistently found [38,39].

Our results support the hypothesis that, in women who are more than 10 years after the menopause and whose trabecular bone density is already substantially reduced, cytokines known to interact with bone resorption are still produced in significantly increased amounts. Other authors have recently confirmed that events taking place in the microenvironment of the bone might be correlated with measurements of cytokines in the blood [40]. This is in agreement with the observation that bone resorption has still an important effect in the determination of bone density in women more than 10 years past the menopause [41]. Confirmation of a high level of bone remodeling in our osteoporotic population by measurement of biochemical markers of resorption and formation would have been interesting. Unfortunately such investigation was not performed in the present study. Circulating levels of IL-1α, IL-1β and IL-6 were recently assayed in serum from healthy women and were not found to correlate with ovarian function or markers of bone remodeling [15] neither were differences observed in circulating levels of these cytokines between osteoporotics and controls [18]. Our results do not disagree with these findings. In fact our two groups were matched for age and ovarian function and our technique of stimulation of whole blood may provide additional information compared to measurement of circulating levels of cytokines [19]. The same comment about divergence in methodologies applies when comparing our results with those from Hustmyer et al. who did not find any significant difference between osteoporotics and non-osteoporotics in IL-1β or IFN-γ production by PBMC stimulated with OKT3, a monoclonal antibody to the T cell-receptor complex, and found no correlation between stimulated production of IL-1β or IFN-γ and bone density [17]. Assessing also cytokine production by stimulated PBMC in a rather small number of osteoporotic ($n = 12$) and healthy ($n = 7$) postmenopausal women, Zarrabeita et al. found no difference in IL-1β, TNF-α or PGE2 between the two populations [42]. Our method of direct stimulation in whole blood was previously compared to the assessment of cytokine production after stimulation of cells isolated from peripheral blood (PBMC) [19]. The methods of purification and handling of the PBMC may artificially modify their reactivity with non-specific interference [43] by removing these cells from their natural surroundings. Our technique of direct activation

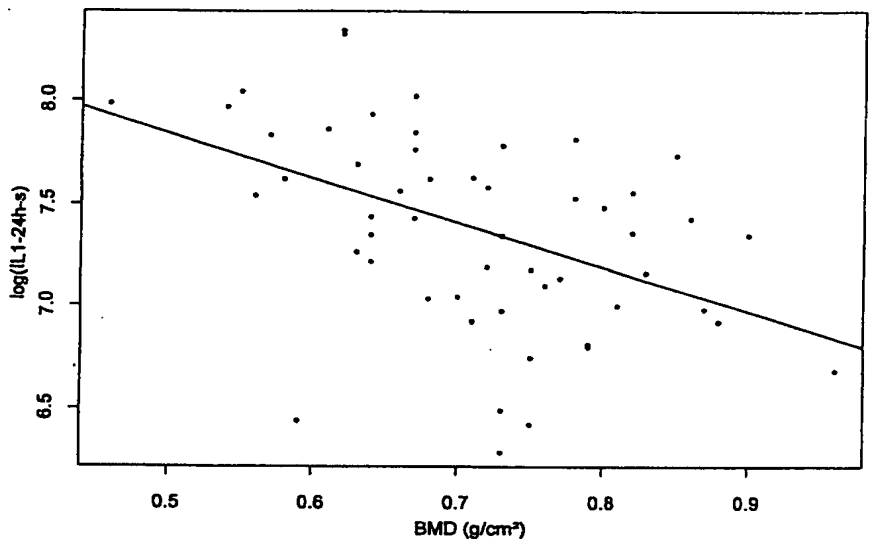

Fig. 1. Relationships between IL-1β production (logarithm of the results, 24-h culture) and BMD of the lumbar vertebral spine in postmenopausal women.

of immune cells in autologous whole blood allows natural cellular interactions and the expression and influence of the local and systemic factors involved in mononuclear cell function [19]. Furthermore, in agreement with our data, Pacifici et al. reported that cultured monocytes from osteoporotics spontaneously produce IL-1 and that there are differences in basal IL-1 production between osteoporotics and non-osteoporotics [26]. Finally, by using reverse transcription/polymerase chain reaction to analyse expression of the mRNAs encoding cytokines in freshly isolated human

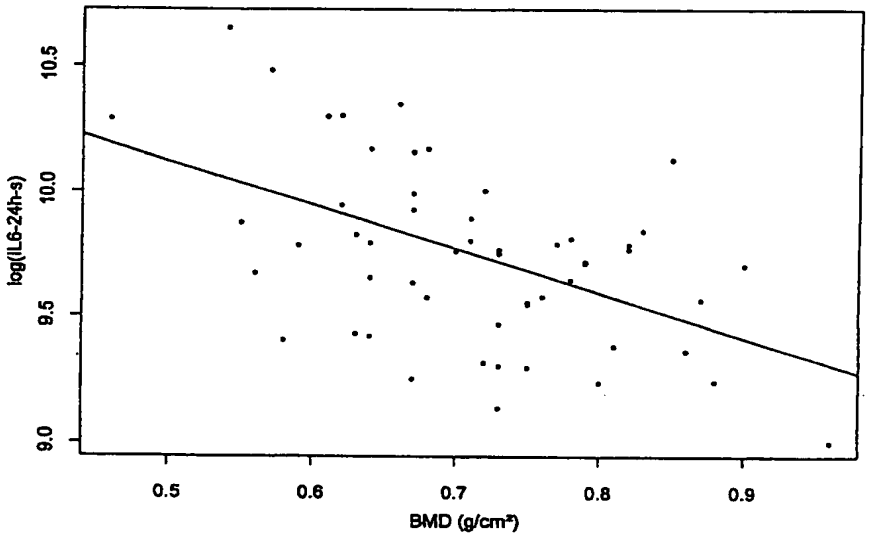

Fig. 2. Relationships between IL-6 production (logarithm of the results, 24-h culture) and BMD of the lumbar vertebral spine in postmenopausal women.

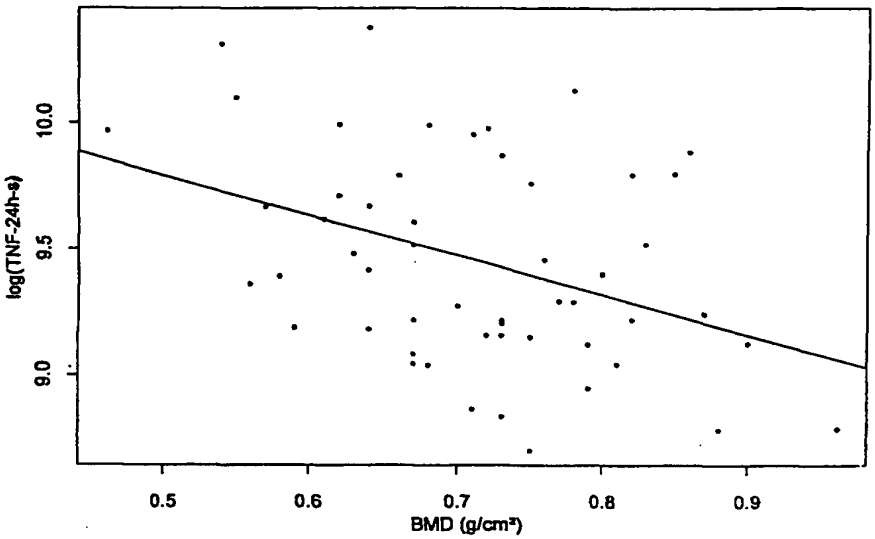

Fig. 3. Relationships between TNF-α production (logarithm of the results, 24-h culture) and BMD of the lumbar vertebral spine in postmenopausal women.

bone biopsy samples, Ralston reported that IL-1α, IL-1β and IL-6 mRNAs were expressed significantly more often in bone samples from postmenopausal women with osteoporotic fractures than in postmenopausal women with normal bone density [16].

From our results, it appears unlikely that LIF, IFN-γ or GM-CSF, even if considered as important endogenous regulators of bone metabolism, still play a predominant role in women with established osteoporosis.

In conclusion, the study of cytokine production by immune cells cultured in autologous whole blood suggest that in women more than 10 years past the menopause and presenting a decrease in lumbar bone density corresponding to the WHO definition of 'osteoporosis', production of IL-1β, IL-6 and TNF-α are still increased compared to controls matched for age and ovarian function, while no differences are reported for IFN-γ, GM-CSF or LIF production. If confirmed, these findings may be of interest in the view of the therapeutic use of cytokine receptor antagonists or cytokine suppressive agents in treatment of established osteoporosis.

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